Cloning and Characterization of a Pectate Lyase Gene from the Soft-Rotting Bacterium *Pseudomonas viridiflava*

C.-H. Liao, K. Sasaki, G. Nagahashi, and K. B. Hicks

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, PA 19118 U.S.A.
Received 30 September 1991. Revised 12 February 1992. Accepted 27 February 1992.

Pseudomonas viridiflava **is a soft-rotting pathogen of harvested vegetables that produces an extracellular pectate lyase (PL) responsible for maceration of plant tissue. A *pel* gene encoding PL was cloned from the genome of strain SJ074 and efficiently expressed in *Escherichia coli*. After a series of deletion subclonings and analysis by transposon mutagenesis, the *pel* gene was located in a 1.2-kb *Pst*I-*Bgl*II genomic fragment. This fragment appears to contain a promoter at the *Pst*I end required for *pel* gene expression. The PL produced by pectolytic *E. coli* clones is identical to those produced by strain SJ074 and by other strains of *P. viridiflava* in terms of molecular weight (42 kDa) and pI (9.7). A mutant of strain SJ074, designated MEI, which had Tn*5* specifically inserted in the *pel* locus was constructed by site-directed mutagenesis. The MEI mutant produced 70- to 100-fold less PL than the wild type and failed to cause tissue maceration in plants. PL production and soft-rot pathogenicity in MEI and in a Pel⁻ mutant previously isolated from strain SF312 were restored to the wild-type level by complementation *in trans* with the cloned *pel* gene. By using the 1.2-kb fragment as a probe, *pel* homologs were detected in four bacteria that are pathologically unrelated to *P. viridiflava*. These include three pathovars of *P. syringae* (pv. *lachrymans*, pv. *phaseolicola*, and pv. *tabaci*) and *Xanthomonas campestris* pv. *malvacearum*. No DNA fragments showing homology to *pel* of *P. viridiflava* were detected in genomic digests prepared from two strains of soft-rot erwinias. The *pel* genes of *Pseudomonas* and *Xanthomonas* appear to be conserved and may have evolved independently from the *pel* family of *Erwinia*.**

Pseudomonas viridiflava (Burkholder) Dowson is a phytopathogenic fluorescent pseudomonad characterized by its ability to macerate plant tissue and by the absence of oxidase and arginine dihydrolase activities (Lelliott *et al.* 1966). This bacterium causes disease symptoms, usually in the form of soft rot, but strains that are capable of producing necrotic and cankerlike lesions in plants have been identified (Billing 1970). On a few occasions, *P. viridiflava* has been shown to be associated with field outbreaks of diseases in horticultural crops (Wilkie *et al.* 973) and with root decay in forage legumes (Leath *et al.* 1989). However, *P. viridiflava* is generally believed to be more important as a postharvest pathogen than as a disease-causing agent in the field. A recent survey shows that this bacterium accounts for more than 10% of bacterial rot of fruits and vegetables at retail and wholesale produce markets (Liao and Wells 1987a).

Pectate lyase (PL) degrades polygalacturonates and other pectic components in plant cell walls by β-*trans* elimination, and is believed to be the principal factor responsible for tissue maceration caused by most strains of soft-rot bacteria including *P. viridiflava*. Two recent studies conducted in our laboratory demonstrate that a relatively simple pectic enzyme system is involved in the elicitation of soft-rot disease by *P. viridiflava*. All eight strains of *P. viridiflava* examined were found to produce a single PL with a pI of 9.7 (Liao 1989). Moreover, no pectin methylesterase or polygalacturonase activities were ever detected in culture filtrates prepared from these strains. The PL produced by *P. viridiflava* is largely secreted into the culture medium, and its synthesis is not regulated by substrate induction or cyclic AMP-mediated catabolite repression (Liao *et al.* 1988). By use of transposon mutagenesis, two genetic loci (*pel* and *out*) that direct the production and secretion of PL in *P. viridiflava* strain SF312 have been identified (Liao *et al.* 1988). Because the *pel* and *out* mutants were unable to induce soft rot in potato tuber slices, the *pel* and *out* genes represent the most critical pathogenicity determinants in *P. viridiflava*. Further investigation of these two pathogenicity determinants would be facilitated by cloning of genomic DNA accounting for their functions. Additionally, the cloned *pel* gene would provide a useful tool for detecting temporarily repressed *pel* genes that may be present in other members of phytopathogenic bacteria such as *P. syringae* van Hall.

Recently, Collmer *et al.* (1991) reported that a *pel* gene cloned from *P. syringae* pv. *lachrymans* expressed very poorly in *E. coli*. We also found that *E. coli* cells carrying a *P. fluorescens* (Trevisan) Migula *pel* gene produced very low levels of PL (Liao 1991). The inefficient gene expression thus appears to be a common problem in the initial cloning of *Pseudomonas* genes when *E. coli* is used as an expression host (Deretic *et al.* 1987). Prior to this study, we have attempted and failed to identify pectolytic *E. coli* clones in genomic libraries constructed from two strains of *P. viridiflava*, including strain SF312 previously used for transposon mutagenesis studies (discussed above). Despite

this, we were able to clone a *pel* gene from an unusual strain (SF074) of *P. viridiflava* by direct selection of pectolytic *E. coli* clones on semisolid pectate medium. Strain SJ074 contains a 160-kb plasmid not found in other strains of *P. viridiflava* (Liao 1988) and is unique for its ability to produce a nonfluorescent orange pigment and its inability to liquify gelatin (Liao and Wells 1987b).

In this paper, we present experimental details on cloning and characterization of the *pel* gene from strain SJ074. We also provide further evidence that the alkaline PL gene of *P. viridiflava* is a key pathogenicity determinant involved in the elicitation of soft-rot disease. In addition, we report detection of *pel* homologs in three pathovars of *P. syringae* and in *Xanthomonas campestris* pv. *malvacearum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. Bacterial strains, plasmids, and bacteriophage that were used in the study are listed in Tables 1 and 2.

Media and culture conditions. Luria broth (LB; GIBCO Laboratories, Grand Island, NY) was used for all liquid cultures. When a solid medium was required, Luria agar (LA) or *Pseudomonas* agar F (Difco Laboratories, Detroit, MI) was used. For *E. coli* strains, minimal salt (MS) solution (Liao 1989) was enriched with yeast extract (0.1%) and Casamino Acid (0.3%; Difco). As needed, glucose, glycerol, and polygalacturonate were added to final concentrations of 0.2, 0.2, and 0.4%, respectively. When required, antibiotics were added at the following concentrations (per milliliter): ampicillin (Ap), 50 μg; kanamycin (Km), 50 μg; tetracycline (Tc), 25 μg; and rifampicin (Rif), 100 μg. A semisolid pectate medium (SSP) prepared as previously described (Liao 1991) was used to assay for pectolytic activity. Unless otherwise indicated, *E. coli* and phytopathogens were grown at 37° and 28° C, respectively.

Cloning, subcloning, and restriction mapping. Total genomic DNA of *P. viridiflava* strain SJ074 was isolated, partially digested with *Sau*3A and fractionated as described by Sambrook *et al.* (1989). Fractions containing 10- to 20-kb fragments were pooled, dialyzed, and further purified on an Elutip-d minicolumn (Schleicher & Schuell, Keene, NH). Ligation of genomic DNA and *Bam*HI-digested dephosphorylated pBR322 was carried out at 14° C for 18 hr in the presence of T4 DNA ligase. Competent cells of *E. coli* were prepared by the $CaCl_2$ procedure (Sambrook *et al.* 1989). Subcloning and restriction mapping were done by standard procedures (Sambrook *et al.* 1989). Deletion derivatives were constructed by digesting the parent plasmid with one endonuclease and ligating the resulting product containing vector DNA with T4 DNA ligase. Desired DNA fragments needed for subcloning were isolated from regular agarose gel by electroelution or from low melting-point agarose gel by the method of Grouse *et al.* (1983). DNA-modifying enzymes used in the study were obtained from Bethesda Research Laboratories (Gaithersburg, MD), New England Biolabs (Beverly, MA), or Boehringer Mannheim Biochemicals (Indianapolis, IN).

Transposon Tn*5* mutagenesis and site-directed mutagenesis. Transposon mutagenesis of pSJB215 with λ467::Tn*5* was carried out by the method of Ruvkun and Ausubel (1981). *E. coli* HB101 carrying pSJB215 was infected with λ467::Tn*5* at a multiplicity of infection of 1, and transductants were selected on LA containing Ap and Km. Plasmid DNA was purified from Ap^r Km^r transductants and reintroduced into *E. coli*, followed by assay on SSP medium for pectolytic activity. Next, plasmid DNAs were isolated from Pel^+ and Pel^- transformants and the positions of Tn*5* insertions in the plasmid were determined by restriction mapping. A Pel^- derivative of pSJB215, designated pSJB2152, which had Tn*5* inserted in the 3′ terminus of the *pel* region was chosen and further characterized. For

Table 1. Bacterial strains used in this study

Designation	Description[a]	Reference or source
Pseudomonas viridiflava		
SJ074	Wild type, carries a plasmid (110 MDa)	Liao and Wells 1987b
SJ074A	Spontaneous Rif^r mutant of SJ074	This study
MEI and MEII	Marker exchange mutants of SJ074A (*pel*::Tn*5*)	This study
SF312	Wild type, produced a single PL (pI 9.7)	Liao *et al.* 1988
MI-4	Pel^- mutant of SF312 (*pel*::Tn*5*)	Liao *et al.* 1988
PJ-08-6A and PJ-08-9	Wild type, isolated from pepper, produce a single PL (pI 9.7)	Liao 1989
Pseudomonas fluorescens (or *P. marginalis*)		
CY091	Produces a PL (pI 10.0), a *pel* gene located in a 1.7-kb fragment	Liao 1991
W51	Produces a pectin lyase instead of PL	A. Kelman
17816, PJ-08-30, SJ-08-2, BC-05-1B, LC-04-2B, and AJ-06-2A	Similar to CY091, all produce an alkaline PL, used for *pel* homology study	Liao 1989
Pseudomonas syringae pathovars		
pv. *lachrymans* (PL785)	Pectolytic on SSP medium	Fett *et al.* 1986
pv. *tomato* (84-86)	nonpectolytic	Fett *et al.* 1986
pv. *phaseolicola* (At)	nonpectolytic	Fett *et al.* 1986
pv. *syringae* (Meyer)	nonpectolytic	Fett *et al.* 1986
pv. *tabaci* (Pt 113)	nonpectolytic	Fett *et al.* 1986
Erwinia chrysanthemi EC16	Produces four PL isozymes	Barras *et al.* 1987
Erwinia carotovora subsp. *carotovora* SR319	Produces at least three PL isozymes	Liao 1989
Xanthomonas campestris pv. *malvacearum*	Pectolytic on SSP medium, isolated from cotton	C. J. Chang
Escherichia coli HB101	Cloning host	BRL

[a] Rif^r = Rifampcin resistance, pI = isoelectric point, PL = pectate lyase, BRL = Bethesda Research Laboratories, SSP = semisolid pectate.

site-directed mutagenesis, a mobilizable plasmid pLA2152 containing *pel*::Tn*5* was constructed by ligating *Eco*RI-digested pSJB2152 with *Eco*RI-digested pLAFR3. pLA2152 was transferred from *E. coli* HB101 into *P. viridiflava* SJ074A by pRK2013-assisted conjugation (Ditta *et al.* 1980), followed by selection on LA plates containing Km and Tc. The marker-exchanged mutants of strain SJ074 were isolated following repeated subculturing in LB containing Km but lacking Tc (Lindgren *et al.* 1986). After five consecutive cycles of subculturing, Km^r Tc^s colonies were selected by replica-plating on LA-Km media containing or lacking Tc.

Preparation and analysis of enzyme samples. The procedures for the quantitative assays of PL (Liao 1991), polygalacturonase (Ried and Collmer 1985), and β-lactamase (Sykes and Matthew 1979) activities have been previously described. Formation of spheroplasts was induced by the method of Witholt *et al.* (1976). Detailed techniques for preparation of enzyme samples from subcellular locations (supernatant, periplasm, and cytoplasm) have been previously described (Liao 1991). The β-lactamases activities in subcellular fractions were monitored to ensure that spheroplasts were properly prepared. When required, enzyme samples were concentrated to contain 0.2–0.3 U of PL activity per microliter by ultrafiltration (PM IO membrane, Amicon Corp., Danvers, MA). One unit of PL activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance (232 nm) at 30° C per minute (Liao 1989). Protein concentrations were measured by the method of Bradford (1976). PL proteins from culture supernantants of *P. viridiflava* strains were purified by ammonium sulfate precipitation and ion-exchange chromatography according to the procedures previously described (Liao 1989). Enzyme samples were analyzed on sodium dodecyl sulfate (SDS)-12% polyacrylamide gels stained with Coomassie blue or on isoelectric focusing gels with pH 3.5–9.5 (PAG plates, Pharmacia-LKB Biotechnology, Piscataway, NJ). Agarose overlay techniques for detection of PL or polygalacturonase activity were done as described (Ried and Collmer 1985). SDS-polyacrylamide gels were prepared and run as previously described (Liao 1991).

Southern hybridization. DNA fragments were modified and labeled by chemical methods, using a Chemiprobe detection kit (FMC Corp., Rockland, ME) according to the manufacturer's instruction. Probe DNA was added at the concentration of 0.5–1.0 μg of DNA per milliliter of hybridization solution. Prehybridization and hybridization were conducted at 42° C in Denhardt's solution containing 50% formamide (Sambrook *et al.* 1989). Southern blots were performed by standard procedures (Sambrook *et al.* 1989). For detection of specific homologous bands, blots were washed in 0.1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate) under high-stringency conditions (65° C, 20 min) as suggested by the manufacturer.

Tissue maceration assays. The ability of bacterial strains to macerate plant tissue was tested on potato tuber slices and detached pepper fruits. General procedures for preparation of testing plant materials and bacterial inocula have been previously described (Liao and Wells 1987a). Maceration zones on pepper fruits in millimeters (diameter) were measured 3 days after incubation at 20° C.

RESULTS

Cloning and analysis of the *pel* gene. *Sau*3A partially digested genomic DNA from *P. viridiflava* strain SJ074

Table 2. Plasmids and bacteriophage used in this study

Designation	Description[a]	Reference or source
pBR322	Cloning and subcloning vector	Balbás *et al.* 1986
pUC18 and pUC19	Subcloning vector	Yanisch-Perron *et al.* 1985
pRZ102	ColE::Tn*5*, used as a Tn*5* probe	Jorgenson *et al.* 1979
pLAFR3	IncP Tc^r Cos^+ rlx^+, used for subcloning and triparental mating	Staskawicz *et al.* 1987
pRK2013	IncP Km^r Tra $RK2^+$ Δ*repRK2 repEI*; helper plasmid used for triparental mating	Ditta *et al.* 1980
pSJB101 to 104	Primary Pel^+ clones; contain *Pseudomonas viridiflava* SJ074 genomic DNA in pBR322, Ap^r	This study
pSJB215	3.8-kb *Sph*I fragment from pSJB101 cloned in pBR322, the *Bgl*II site of the insert proximal to the vector Tc^r promoter, Ap^r Pel^+	This study
pSJB225	Same as pSJB215, except that the fragment was cloned in the opposite orientation, Ap^r Pel^+	This study
pSJBB610	2.3-kb *Pst*I-*Bam*H fragment from pSJB215 cloned in pUC19, the *Pst*I site placed downstream of the vector *lac* promoter, Ap^r Pel^+	This study
pSJB620	Same as pSJB610, except that the 2.3-kb fragment cloned in pUC18, the *Bam*HI site placed downstream of the *lac* promoter, Ap^r Pel^+	This study
pSJB710	1.2-kb *Pst*I-*Bgl*II fragment from pSJB215 cloned in pUC19, the *Pst*I site proximal to the *lac* promoter, Ap^r Pel^+	This study
pSJB720	Same as pSJB710, except that the 1.2-kb fragmet cloned in pUC18, the *Pst*I site distal to the *lac* promoter, Ap^r Pel^+	This study
pSJB2152	λ-Mediated Tn*5* insertion mutant of pSJB215, Tn*5* inserted in 3′ end of the *pel* region Ap^r Km^r Pel^-, *pel*::Tn*5*	This study
pLA215	A chimeric plasmid constructed by ligating *Eco*RI-digested pSJB215 with *Eco*RI-digested pLAFR3, Ap^r Tc^r Pel^+	This study
pLA2152	Similar to pLA215, constructed by ligating *Eco*RI-digested pSJB2152 with *Eco*RI-digested pLAFR3, Ap^r Tc^r Km^r Pel^-, *pel*::Tn*5*	This study
Bacteriophage λ467::Tn*5*	λ6221 *rex*::Tn*5* c1857, oam 29, pam 80, used for Tn*5* mutagenesis	Ruvkun and Ausubel 1981

[a] Ap^r, Km^r, Tc^r = Resistance to ampicillin, kanamycin, and tetracycline, respectively. Pel^+ = pectolytic, Pel^- = nonpectolytic.

was ligated with *Bam*HI-restricted and dephosphorylated pBR322. The ligation sample was used to transform *E. coli* HB101 followed by selection on SSP plates containing Ap. Of 931 Ap^r transformants examined, four showed pectolytic activity on SSP plates. Restriction analysis of recombinant plasmids isolated from these four pectolytic clones (pSJB101 to 104) revealed that each plasmid contained an insert in the 10–12 kb size range. None of these four recombinant plasmids yielded identical *Sph*I restriction patterns, indicating that they were derived from independent insertions. All four pectolytic clones contained a common 3.8-kb *Sph*I and a common 1.2-kb *Pst*I-*Bgl*II region of the *P. viridiflava* genome. Pectolytic enzymes produced by all four *E. coli* clones were determined to be PLs by the 232-nm absorbance assay. No polygalacturonase activity was detected in enzyme samples from pectolytic clones either by colorimetric methods or by overlay activity stains. PLs produced by all four *E. coli* clones were located largely (over 79%) in the periplasm (Table 3). Activity-stained isoelectric focusing gels revealed that all four *E. coli* clones and *P. viridiflava* strain SJ074 produce one single PL with an approximate pI of 9.7, which was distinguishable from the PLs produced by *P. fluorescens* and *Erwinia chrysanthemi* (Fig. 1).

Table 3. Production of pectate lyase by pectolytic *Escherichia coli* clones

	Total activity[a] ($U/10^{10}$ cells)	% Total activity		
		Culture fluid	Periplasm[b]	Cytoplasm
E. coli				
pSJB101	2.0	11	82	7
pSJB102	4.0	6	79	15
pSJB103	1.4	9	85	6
pSJB104	5.5	10	89	1

[a]Grown in minimal salt medium containing 0.3% Casamino Acids and 0.4% glycerol. One unit of activity is defined as the amount of enzyme which causes an increase of 1.0 absorbance (232 nm) at 30° C per minute. The values shown represent an average of three independent experiments.
[b]84–91% of total β-lactamase activity was detected in the preiplasmic fraction in *E. coli* cells.

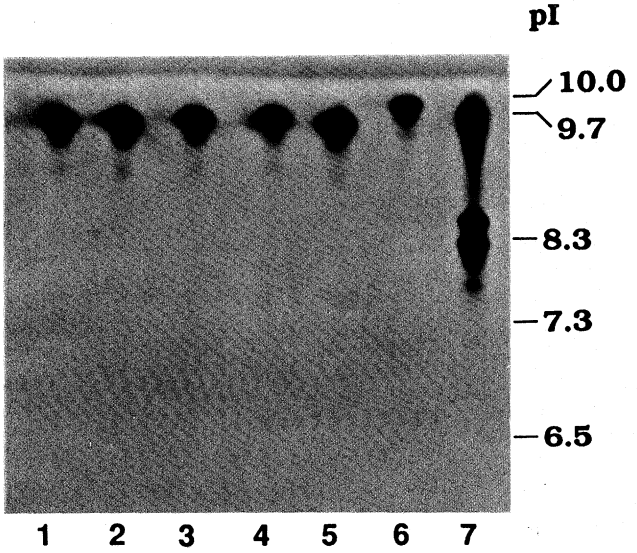

Fig. 1. Analysis of pectate lyase (PL) from *Escherichia coli* clones by isoelectric-focusing electrophoresis in ultrathin-layer polyacrylamide gel and by overlay enzyme-activity stain. Lanes 1–4, PL sample from *Escherichia coli* containing pSJB101 (lane 1), pSJB102 (lane 2), pSJB103 (lane 3), and pSJB104 (lane 4). Lanes 5–7, PL samples from culture fluids of *Pseudomonas viridiflava* SJ074 (lane 5), *P. fluorescens* CY091 (lane 6), and *Erwinia chrysanthemi* EC16 (lane 7).

To further locate the *pel* gene, the primary clone pSJB101 was digested with *Sph*I and subjected to deletion subcloning. Deletion derivatives of pSJB101 indicated that the common 3.8-kb *Sph*I fragment found in all four primary clones was sufficient to confer pectolytic activity. When this fragment was transferred into pBR322 in either orientation, the resulting plasmids (pSJB215 and pSJB225) in *E. coli* conferred similar pectolytic activity. This indicates that the 3.8-kb fragment likely contains a promoter active in *E. coli*. Subfragments of the 3.8-kb *Sph*I fragment generated by *Kpn*I, *Sal*I, *Pst*I, and *Bgl*II were introduced into pBR322, pUC18, or pUC19, and the resulting plasmids were introduced into *E. coli* and tested for pectolytic activity. The results, summarized in Figure 2, indicate that the *pel* gene is totally contained within a 1.2-kb *Pst*I-*Bgl*II region. To further confirm the location of *pel* region in the 3.8-kb fragment, pSJB215 was mutagenized with λ467::Tn*5*. Four Pel^- derivatives of pSJB215, designated pSJB2151, pSJB2152, pSJB2153, and pSJB2154, were isolated and analyzed with restriction endonucleases *Pst*I and *Bgl*II. The positions of Tn*5* insertions in these four Pel^- derivatives were all located within the 1.2-kb *Pst*I-*Bgl*II *pel* region (Fig. 2).

Concentrated periplasmic fluids from *E. coli* clones containing pSJB710 or pSJB101 were analyzed by SDS-polyacrylamide gel electrophoresis and by overlay activity stain. A protein band (42 kDa) showing PL activity as confirmed by the overlay activity stain was detected in periplasmic fluids prepared from *E. coli* cells containing pSJB710 or pSJB101 but not in that prepared from cells containing pUC19. This result indicates that the 11-kb insert

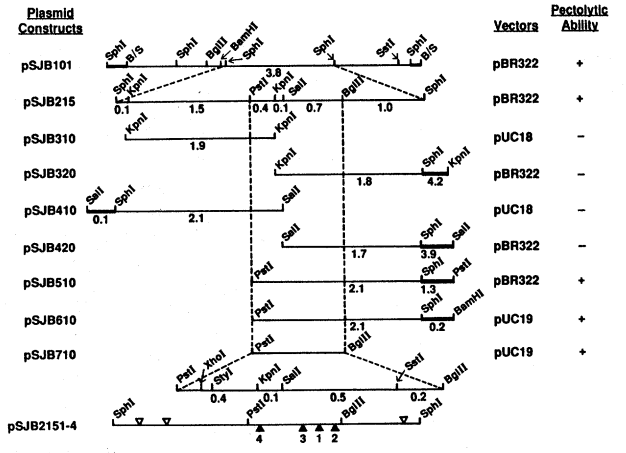

Fig. 2. Restriction map of cloned genomic regions and positions of Tn*5* insertions in a *pel*-containing fragment. Thick lines denote vector DNAs and thin lines represent *Pseudomonas viridiflava* genomic regions. The B/S indicates a site created by a ligation between the *Bam*HI and *Sau*3A cohesive ends. The length of DNA fragment is shown in kilobase under the line. The positions of Tn*5* insertion sites in pSJB215 are shown in open and solid triangles, which denote the Pel^+ and Pel^- phenotype, respectively. The *pel* transcription, as determined by the induction study, is directed from the *Pst*I end of the *pel* fragment in pSJB710.

in the primary clone pSJB101 encoded a single *pel* gene. The molecular mass of PL produced by pectolytic *E. coli* clones appeared to be close to its counterparts produced by *P. viridiflava* strains SJ074 and SF312 (Fig. 3). As reported before (Liao 1989), the PL protein from *P. viridiflava* is slightly larger than that from *P. fluorescens* (lanes 4–6, Fig. 3).

Direction of *pel* transcription. The 1.2-kb *Pst*I-*Bgl*III fragment containing the *pel* gene was introduced into pUC18 and pUC19 with one specific end of the insert adjacent to the vector *lac* promoter. When the *Pst*I end of the fragment was placed immediately downstream of the *lac* promoter in pUC19, the resulting plasmid pSJB710 in *E. coli* directed production of 6.3–10.0 U of PL per 10^{10} cells. However, when the *Pst*I end was inserted into pUC18 in a position distal to the *lac* promoter, the resulting plasmid pSJB720 directed production of relatively smaller amounts of PL (2.9–5.1 U per 10^{10} cells) in *E. coli*. Furthermore, production of PL by *E. coli* containing pSJB710 was induced two- to sevenfold in the presence of isopropyl thiogalactoside (IPTG). Induction of PL production by IPTG was not observed with *E. coli* containing pSJB720. This result in combination with those summarized in Table 4 indicates that: 1) the direction of *pel* transcription is initiated from the *Pst*I end of the *pel* fragment; 2) the cloned 1.2-kb *pel* fragment likely contains a promoter at the *Pst*I end, responsible for self-expression of the *gel* gene in pSJB720; and 3) expression of the cloned *pel* gene in *E. coli* is not affected by the the type of carbon source included in the medium.

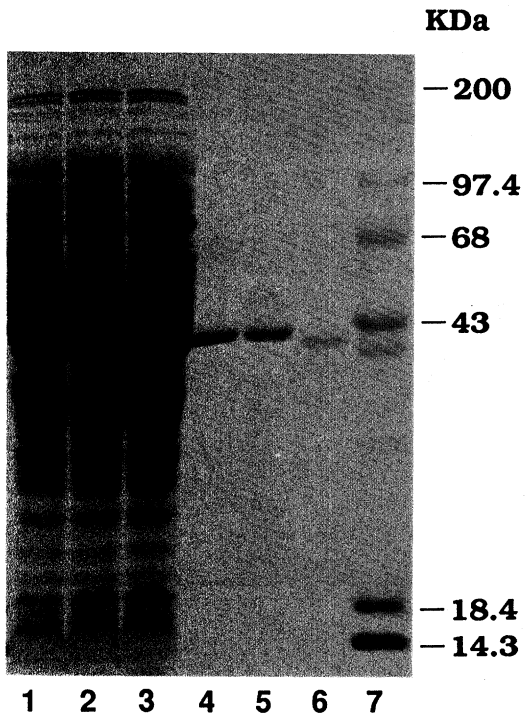

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of pectate lyase (PL) proteins. Lanes 1–3, concentrated periplasmic fluids from *Escherichia coli* carrying pUC19 (lane 1), pSJB101 (lane 2), and pSJB710 (lane 3). Lanes 4–6 purified PLs from *Pseudomonas viridiflava* strains SJ074 (lane 4) and SF312 (lane 5), and from *P. fluorescens* strain CY091 (lane 6). Molecular weight markers (lane 1) were: myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and lysozyme (14.3 kDa).

Isolation of *pel*-defective mutants. *P. viridiflava* strain SJ074A containing pLA2152 (*pel*::Tn*5*) was grown in the medium containing Km but lacking Tc. After five consecutive cycles of subculturing, two Km^r Tc^s colonies, designated MEI and MEII, were randomly selected and further characterized. Both MEI and MEII mutants lacked pLA2152 and showed no pectolytic activity on SSP medium after incubation at 28° C for 4 days. However, when grown in liquid medium, these two mutants produced a residual amount of PL (0.01 U/10^{10} cells), which was about 70- to 100-fold less than that produced by the wild type (0.80 U/10^{10} cells). Despite the residual PL activity detected, MEI and MEII mutants were unable to induce soft rot in potato tuber slices and in pepper fruits. To confirm that these two *pel*-defective mutants resulted from specific insertion of Tn*5* into the *pel* locus, genomic digests prepared from the wild type and mutants were analyzed with the *pel*- and Tn*5*-specific probes by Southern hybridization. The functional *pel* locus of the wild type was detected in a 17.8-kb *Bam*HI fragment and in a 1.2-kb *Pst*-*Bgl*II fragment (Fig. 4A, lanes 1 and 4). No Tn*5* sequences were found in the genomic digest of the wild type (Fig. 4B, lanes 1 and 4). In genomic digests prepared from mutants MEI and MEII, the genomic region containing 5′ end of the *pel* (1.1 kb) and adjacent Tn*5* sequences was detected by the *pel* probe (Fig. 4A) or the Tn*5* probe (Fig. 4B), in a 1.6-kb *Pst*I-*Bgl*II fragment (lanes 2 and 3) and in a 5.4-kb *Bam*HI fragment (lanes 5 and 6). The region containing 3′ end of the *pel* (0.1 kb) and adjacent Tn*5* sequences was detected by the Tn*5* probe, but not by the *pel* probe, in a 0.7-kb *Pst*I-*Bgl*II fragment (Fig. 4B, lanes 2 and 3) and in an 18-kb *Bam*HI fragment (Fig. 4B, lanes 5 and 6). Failure to detect the fragments containing the 3′ end of the *pel* region by the *pel* probe was presumably due to the limited *pel* sequence (0.1 kb) present in these fragments. This result is in a good agreement with the data obtained from restriction analysis of pLA2152, which show that the position of Tn*5* in the 1.2-kb *Pst*I-*Bgl*II *pel* region is at about 0.1 kb from the *Bgl*II site.

Restoration of soft-rot pathogenicity by complementation. Plasmid pLA215 containing a functional *pel* gene from strain SJ074 was transferred into MEI and into a Pel^-

Table 4. Production of pectate lyase by pectolytic *Escherichia coli* clones grown in media containing various carbon sources

		PL act. (U/10^{10} cells) in medium containing[a]		
E. coli	IPTG	Glucose	Glycerol	Glycerol + polygalacturonate
pSJB215	−	2.1	3.3	4.3
pSJB610	−	4.2	6.8	6.7
pSJB620	−	2.7	4.2	4.5
pSJB710	+	12.4	56.4	68.5
	−	6.3	8.3	10.0
pSJB720	+	3.1	4.2	5.8
	−	2.9	5.1	4.8

[a]The carbon source and isopropyl thiogalactoside (IPTG) were added to 0.4% and 1 mM, respectively. Only the activity in periplasm was determined. The values shown represents an average of three experiments. +: presence; −: absence.

mutant MI-4 derived from strain SF312 (Liao *et al.* 1988) by triparental matings. Twenty-five Km^r Tc^r colonies from each mating were randomly selected and tested for pectolytic and tissue-macerating abilities. All 25 Km^r Tc^r colonies containing pLA215 showed the same degree of pectolytic activity on SSP medium as the wild type. When assayed on plants, the merodiploid strains (*pel*$^+$/*pel*::Tn*5*) were able to macerate potato tubers and pepper fruits. Maceration zones on pepper fruits caused by the wild type or by the merodiploid strain were in the range of 3–7 mm in diameter after incubation at 20° C for 3 days.

Detection of *pel* homologs in *P. syringae* and *X. campestris*. *Eco*RI-generated genomic digests from 18 strains of phytopathogenic bacteria were examined for the presence of *pel* homologous sequences by using the 1.2-kb cloned *pel* gene as a probe. Fragments showing strong hybridization with the *pel* probe were detected in genomic digests prepared from four strains of *P. viridiflava* (Fig. 5A, lanes 1A) and seven strains of *P. fluorescens* (Fig. 5B, lanes 1–7). Fragments showing weak hybridization with the *pel* probe were also observed in genomic digests prepared from one strain each of *P. s.* pv. *lachrymans*, *P. s.* pv. *phaseolicola*, *P. s.* pv. *tabaci* (Fig. 5A, lanes 5, 7, and 9), and *X. campestris* pv. *malvacearum* (Fig. 5B, lane 10). Sequences homologous to the *pel* gene from *P. viridiflava* SJ074 were not detected in genomic digests prepared from *P. syringae* pv. *tomato*, *P. syringae* pv. *syringae*, *E. chrysanthemi* Burkholder *et al.*, *E. carotovora* (Jones) Bergey *et al.* subsp. *carotovora*, and from *P. fluorescens* strain W51, which had been shown to produce a pectin lyase instead of PL (Schlemmer *et al.* 1989).

DISCUSSION

We report here cloning and efficient expression in *E. coli* of a *pel* gene from *P. viridiflava* strain SJ074. Previously, it has been shown that some of *Pseudomonas* genes are difficult to express in *E. coli* (Deretic *et al.* 1987; Collmer *et al.* 1991 and Liao 1991). The *pel* genes from other strains of *P. viridiflava* also appear to express very poorly in *E. coli*. So far, we have been unable to clone the *pel* genes from *P. viridiflava* strains SF312 and PJ-08-6A by direct selection of pectolytic *E. coli* clones in the genomic libraries of these two strains. However, by using the *pel* gene of strain SJ074 as a probe, we have recently identified a Pel^+ *E. coli* clone in the library of strain PJ-08-6A (C.-H. Liao, unpublished). The *pel* gene of strain PJ-08-6A is able to

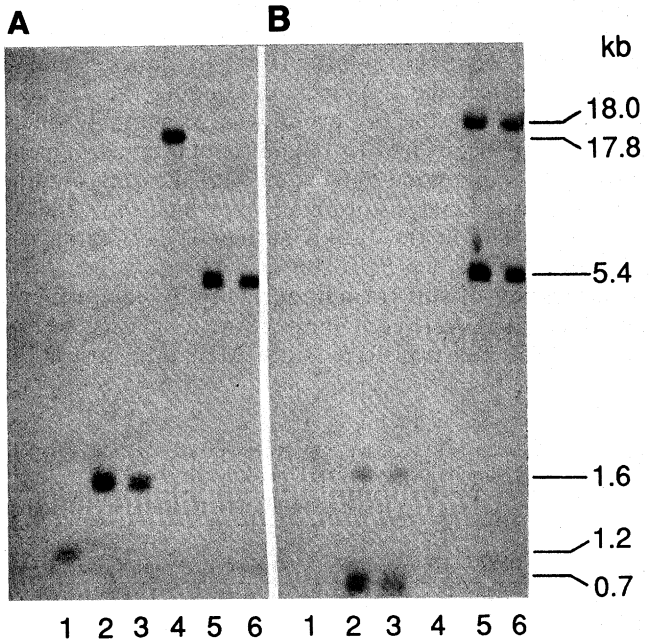

Fig. 4. Southern hybridization analysis of Tn*5* insertions in marker-exchanged mutants. Genomic DNAs from the wild type (SJ074A) and mutants (MEI and MEII) were digested with *Pst*I-*Bgl*II or *Bam*HI, and probed with the 1.2-kb *pel*-specific fragment or with Tn*5*-specific pRZ102. Digested DNA samples in the blot were: lane 1, SJ074A/*Pst*I-*Bgl*II; lane 2, MEI/*Pst*I-*Bgl*II; lane 3, MEII/*Pst*I-*Bgl*II; lane 4, SJ074A/*Bam*HI, lane 5, MEI/*Bam*HI and lane 6, MEII/*Bam*HI. Blots A and B were prepared in the same way except that blot A was probed with the *pel* fragment and blot B with pRZ102 (Tn*5*).

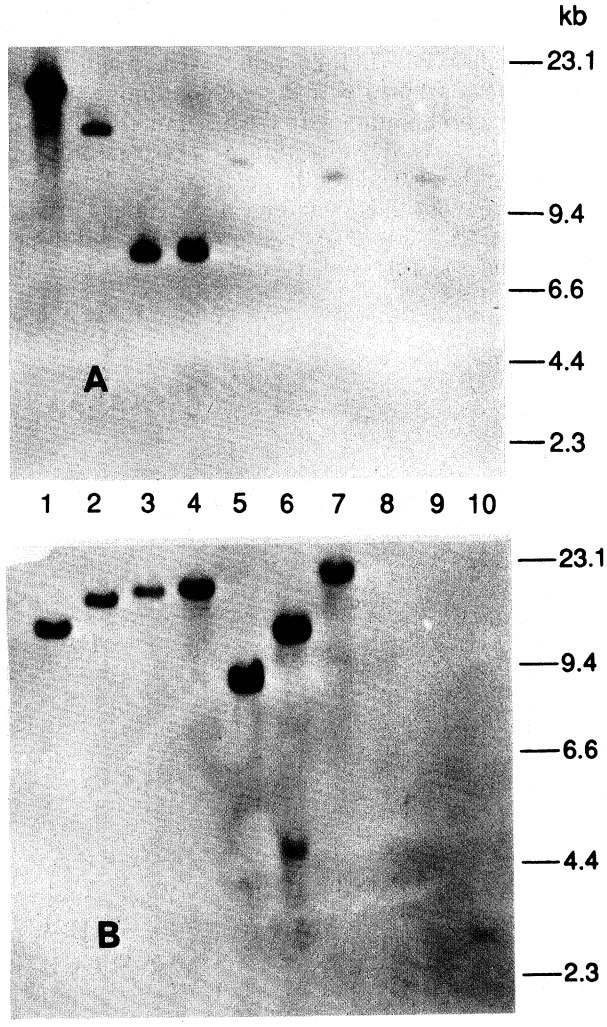

Fig. 5. Dection of *pel* homologs in phytopathogenic bacteria. **A,** Lanes 1A; *Pseudomonas viridiflava* strains SJ074 (lane 1), SF312 (lane 2), PJ-08-6A (lane 3), and PJ-08-9 (lane 4); lane 5, *P. syringae* pv. *lachrymans*; lane 6, *P. s.* pv. *tomato*; lane 7, *P. s.* pv. *phaseolicola*; lane 8, *P. s.* pv. *syringae*; lane 9, *P. s.* pv. *tabaci* and lane 10, *Erwinia chrysanthemi* EC16. **B,** Lanes 1–8; *P. fluorescens* strains CY091 (lane 1), ATCC 17816 (lane 2), PJ-08-30 (lane 3), SJ-08-2 (lane 4), BC-05-1B (lane 5), LC-04-2B (lane 6), AJ-06-2A (lane 7), and W51 (lane 8); lane 9, *E. carotovora* subsp. *carotovora*, and lane 10, *Xanthomonas campestris* pv. *malvacearum*.

direct synthesis of high levels of PL in two Pel⁻ mutants (MI-4 and MEI) of *P. viridiflava*, but not in *E. coli*. The reason why the transcription-translation machinery of *E. coli* can recognize the *pel* of strain SJ074 but not the *pel* of strain PJ-08-6A is presently unclear. Deretic *et al.* (1987) have shown that the *algD* promoter of *P. aeruginosa* does not follow the typical -10/-35 consensus pattern of *E. coli* promoters. It awaits to be determined if the differential expression of *P. viridiflava pel* genes in *E. coli* is due to the structural difference in *pel* promoters.

By use of transposon mutagenesis, we have previously demonstrated that inability of the Pel⁻ mutant (MI-4) of strain SF312 to produce PL is accompanied by the loss of its ability to induce soft rot in harvested vegetables (Liao *et al.* 1988). In this study, we have constructed another Pel⁻ mutant in strain SJ074 by marker-exchange mutagenesis. Like the Tn*5*-induced Pel⁻ mutant (MI-4) of strain SF312, the marker-exchanged mutant (MEI) of strain SJ074 was also unable to induce soft rot in potato tubers and in pepper fruits. Furthermore, we found that the soft-rotting ability of both MI-4 and MEI mutants could be restored to the wild-type level by complementation *in trans* with a *pel* gene cloned from strain SJ074. This result indicates that the alkaline PL encoded on the cloned *pel* gene is the principal or sole pectic enzyme of *P. viridiflava* required for maceration of plant tissue and for induction of soft-rot disease. Previously, we have analyzed the IEF profile of PLs produced by eight strains of *P. viridiflava* and found that each strain produces a single PL with an approximate pI of 9.7 (Liao 1989). The gene coding for this PL in *P. viridiflava* appears to be well conserved. In this study, we have analyzed the genomic digests prepared from four strains of *P. viridiflava* by Southern hybridization. Results (Fig. 5) show that the *pel* gene of each strain is contained in a single *Eco*RI genomic fragment.

Although marker-exchanged mutants MEI and MEII were unable to cause soft rot in potato tubers and in pepper fruits, they still produced a trace amount of PL in cultures. The origin of this residual PL is presently obscure. Data from Southern analysis of the *pel* locus in MEI and MEII mutants (Fig. 4) and from restriction analysis of pLA2152 (*pel*::Tn*5*) indicate that the mutation is caused by a specific insertion of Tn*5* into the 1.2-kb *Pst*I-*Bgl*II *pel* region. It is possible that the residual PL may have resulted from the insertion of Tn*5* into a regulatory region adjacent to the 3′ end of *pel* structural sequences. Alternatively, it is also possible that the residual PL activity represents the function of a truncated PL resulting from the insertion of Tn*5* into the 3′ end of *pel*-coding sequences. All of the data presented in this study and elsewhere (Liao *et al.* 1988 and Liao 1989) suggest that *P. viridiflava* produces an alkaline PL responsible for tissue maceration. So far, there is no evidence that this organism may contain a second *pel* gene, accounting for the residual PL activity of MEI and MEII mutants. It should be noted, however, that Collmer *et al.* (1991) recently have identified a set of *pel* genes in *E. chrysanthemi* that are inducible only by plant tissue extracts. The possibility that the residual PL activity of MEI and MEII mutants may result from the action of a plant-inducible *pel* gene cannot be totally excluded. Because *P. viridiflava* rarely causes diseases in growing plants and is generally considered an opportunistic post-harvest pathogen, this organism probably does not contain a set of plant-inducible genes required for specific interactions with host genes plants in the field. The residual PL activity of MEI and MEII mutants is therefore more likely derived from a mutation in the *pel* gene already identified in the study.

Expression of the pectolytic phenotype in fluorescent pseudomonads is a variable character and can be influenced by a number of factors in culture media and in plants (Zucker and Hankin 1970). The *pel*-specific DNA probe would provide a useful tool for detection of temporarily repressed *pel* genes that may be present in plant-associated bacteria. We have previously reported the presence of *pel* homologs in two nonpectolytic strains of *P. putida* originally isolated for biocontrol applications (Liao 1991). By using the *pel* gene of *P. viridiflava* SJ074 as a probe, we have detected *pel* homologs in two pathovars of *P. syringae* (pv. *phaseolicola* and pv. *tabaci*) that have not yet been reported to produce pectic enzymes under laboratory conditions. It remains to be determined whether the *pel* homologs detected in these two pathovars are active genes associated with plant pathogenesis. The presence of *pel* homologs in other pathovars of *P. syringae* has also been observed by Collmer and associates (1991). In this study, we found that the *pel* gene of *P. viridiflava* showed no homology with the genomic digests prepared from two strains of soft-rot erwinias. Nevertheless, Fennington and Hughes (1990) recently reported that a 2.3-kb *pel* fragment from *E. carotovora* subsp. *atroseptica* hybridized weakly with the *P. marginalis* chromosome only under low-stringency conditions. While the *pel* genes in pectolytic fluorescent pseudomonads appear to be conserved, they may be distantly related to the *pel* family of *Erwinia* (Kotoujansky 1987).

ACKNOWLEDGMENTS

We thank A. K. Chatterjee, A. Kelman, C. J. Chang, M. J. Haas, and W. F. Fett for providing some of the bacterial strains used in the study. We also thank D. Solaiman, D. E. McCallus, and W. F. Fett for critical reading of the manuscript.

LITERATURE CITED

Balbás, P., Soberón, X., Merono, E., Zurita, M., Lomeli, H., Valle, F., Flores, N., and Bolivar, F. 1986. Plasmid vector pBR322 and its special-purpose derivatives: A review. Gene 50:3-40.

Barras, F., Thurn, K. K., and Chatterjee, A. K. 1987. Resolution of four pectate lyase structural genes of *Erwinia chrysanthemi* (EG16) and characterization of the enzymes produced in *Escherichia coli*. Mol. Gen. Genet. 209:319-325.

Billing, E. 1970. *Pseudomonas viridiflava* (Burkholder; 1930; Clara 1934). J. Appl. Bacteriol. 33:492-500.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Collmer, A., Bauer, D. W., He, S. Y., Lindberg, M., Kelemu, S., Rodriguez-Palenzuela, P., Burr, T. J., and Chatterjee, A. K. 1991. Pectic enzyme production and bacterial plant pathogenicity. Pages 15-21 in: Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Crouse, G. F., Frischauf, A., and Lebrach, H. 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages. Methods Enzymol. 101:787-89.

Deretic, V., Gill, J. F., and Chakrabarty, A. M. 1987. *Pseudomonas* infection in cystic fibrosis: Nucleotide sequence and transcriptional

regulation of *atgD* gene. Nucleic Acids Res. 15:4567-4581.
Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.
Fennington, G. H., and Hughes, T. A. 1990. *Erwinia pel* gene homology survey in selected bacteria. Plant Soil 125:285-287.
Fett, W. F., Osman, S. F., Fishman, M. L., and Siebles, T. S. 1986. Alginate production by plant-pathogenic pseudomonads. Appl. Environ. Microbiol. 52:466-473.
Jorgenson, R. A., Rothstein, S. J., and Rezinikoff, W. S. 1979. A restriction enzyme cleavage map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. 177:65-72.
Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot erwinias. Annu. Rev. Phytopathol. 25:405-430.
Leath, K. T., Lukezic, F. L., Pennypacker, B. W., Kendall, W. A., Levine, R. G., and Hill, R. R., Jr. 1989. Interaction of *Fusarium avenacearum* and *Pseudomonas viridiflava* in root rot of red clover. Phytopathology 79:436-440.
Lelliott, R. A., Billing, E., and Hayward, A. C. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.
Liao, C.-H. 1988. Presence of extrachromosal elements in the soft-rotting bacterium *Pseudomonas viridiflava*. Phytopathology 78:1552.
Liao, C.-H. 1989. Analysis of pectate lyase produced by soft rot bacteria associated with spoilage of vegetables. Appl. Environ. Microbiol. 55:1677-1683.
Liao, C.-H. 1991. Cloning of a pectate lyase gene *pel* from *Pseudomonas fluorescens* and detection of sequence homologous to *pel* in *Pseudomonas viridiflava* and *Pseudomonas putida*. J. Bacteriol. 173:4386-4393.
Liao, C.-H., and Wells, J. M. 1987a. Diversity of pectolytic fluorescent pseudomonads causing soft rots of fresh vegetables at produce markets. Phytopathology 77:673-677.
Liao, C.-H., and Wells, J. M. 1987b. Properties of yellow (orange) pigmented strains of soft-rot bacteria in the genus *Xanthomonas, Pseudomonas, Cytophaga,* and *Erwinia*. Pages 578-583 in: Plant Pathogenic Bacteria, E. L. Civerolo, A. Collmer, R. E. Davis, and A. G. Gillaspie, eds. Martinus Nijhoff Publishers, The Hague.
Liao, C.-H., Hung, H. Y., and Chatterjee, A. K. 1988. An extracellular pectate lyase is the pathogenicity factor of the soft-rotting bacterium *Pseudomonas viridiflava*. Mol. Plant-Microbe Interact. 1:199-206.
Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "phaseolicola" controls pathogenicity of bean plants and hypersensitivity on non-host plants. J. Bacteriol. 68:512-522.
Ried, J. L., and Collmer, A. 1985. Activity stain for rapid characterization of pectic enzymes in isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. Appl. Environ. Microbiol. 50:615-622.
Ruvkun, G. B., and Ausubel, F. M. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) 289:85-88.
Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Schlemmer, A. F., Ware, C. F., and Keen, N. T. 1987. Purification and characterization of a pectin lyase produced by *Pseudomonas fluorescens* W51. J. Bacteriol. 169:4493-4498.
Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned virulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.
Sykes, R. B., and Matthew, M. 1979. Detection assay and immunology of β-lactamases. Pages 17-49 in: β-Lactamases. M. T. Hamilton-Miller and J. T. Smith, eds. Academic Press, New York.
Wilkie, J. P., Dye, D. W., and Watson, D. R. W. 1973. Further hosts of *Pseudomonas viridiflava*. N.Z. J. Agric. Res. 16:315-323.
Witholt, B., Boekhout, M., Brock, M., Kingma, J., Van Heerikhuizen, H., and De Leij, L. 1976. An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. Anal. Biochem. 74:160-170.
Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene 33:103-119.
Zucker, M., and Hankin, L. 1970. Regulation of pectate lyase synthesis in *Pseudomonas fluorescens* and *Erwinia carotovora*. J. Bacteriol. 104:13-18.